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NF- κ B ACTIVATION INHIBITORS, AND THEIR

PHARMACEUTICAL USES

The present invention has for its object the use of biological inhibitors of NF- κ B, in the field of treating cancers, and more particularly malignant hemopathies or solid tumors.

5 Numerous tumoral cells have developed sophisticated mechanisms permitting them to resist the effect of certain agents used in anti-cancer chemotherapy. One of the countermeasures at present developed by clinicians is the increase of the dosage of these medications, with the result of aggravating the side effects observed in
10 the patients. Thus, for example, most of the leukemias and certain lymphomas are treated by the administration of anthracyclines (daunomycin, dauxorubicin) whose toxicity is manifest in the vital functions (hepatic, cardiac...) (Gauthier, PH, 1987, Gas Med Fr, 94:43-49).

The mechanism of action of the medications has been well
15 studied and has essentially led to the death of tumor cells by apoptosis (Hannum YA, Blood, 89:1845-1853). To avoid apoptosis, the cells use a category of proteins encoded by genes called *multidrug resistant genes* (MDR) which permit them to control the intake or outflow of various molecules (Pastan I, Gottesman MM, 1991, Annu Rev Med, 42:277-286). In

the case of anti-cancer agents, these are actively evacuated by means of P-glycoprotein (P-gp), produced by the *MDR1* gene.

As all genes, the expression of the *MDRs* is controlled by different nuclear factors. Thus, it has been recently shown that the *MDR1* gene has in its regulatory portion binding sites of the NF- κ B factor (Zhou G, Kuo MT, 1997, J Biol Chem, 272:15174-15183). This nuclear factor, which moreover plays a considerable role in numerous inflammatory situations (Barnes PJ, Karin M, 1997, N Engl J Med, 336:1066-1071) participates in the activation of the *MDR1* gene.

Several recent works have established a connection between the inhibition and the activation of NF- κ B and the potentialization of apoptosis. In the first reported experiments (Wang CY et coll., 1996, Science, 272:784-786, Van Antwerp DJ et coll., Science, 272:787-789) the authors have validated their data by using genetically manipulated lines to obtain the inhibition or the overexpression of NF- κ B activity. Thus, this does not permit their direct use in therapeutic applications.

In another study, the authors have tested the effects of different protease inhibitors preventing the activation of NF- κ B (pyrrolidine dithiocarbamate, *N*-tosyl-L-lysyl chloromethylcetone, *N*-acetyl cysteine) on a line of murine macrophages (Mannick EE et coll., 1997, Mediators of Inflammation, 6:225-232). The authors of this article conclude there is a possible connection between NF- κ B inhibition and the induction of apoptosis of the inflammatory and immune cells.

Finally, another approach based on inhibition of the

inflammatory effects of NF- κ B, consists in overexpressing the natural inhibitor of NF- κ B, the I κ B molecule, by gene therapy (Makarov SS et coll., 1997, Gene Ther, 4:846-852). This technology is also in the state of development because of the complexity and the vectorization necessary
5 for its good operation.

The present invention results from the discovery by the inventors of new effects of the human growth hormone (HGH), also called somatotropin, namely, on the one hand that HGH, and other compounds
10 connected specifically to the transmembrane receptors of class I cytokines, are inhibitors of the activation of NF- κ B by a cytotoxic molecule, and, on the other hand, that HGH, and other above-mentioned compounds, permit potentiating the effects of cytotoxic molecules and hence reducing the concentrations of these latter in the field of
15 therapeutic treatments.

First of all, the inventors have observed that the human monocytes respond less to a stimulation by lipopolysaccharides (LPS) when they are cultivated in the presence of exogenous recombinant HGH. The inventors have accordingly concluded that HGH inhibits the activation of
20 NF- κ B after stimulation by LPS (Haefner A et coll., 1997, J Immunol, 158:1310-1314).

Then, the inventors discovered that the human monocytes died after bridging (or engagement) of the surface molecule APO1/CD95/Fas, and have shown that HGH decreases the death mediated through the molecule

Fas, by increasing the synthesis of an antiapoptogenic proto-oncogene Bcl-2.

Finally, the inventors have studied the effects of HGH on the -TNF response, because Fas and the p55 receptor of the α -TNF belong to the same family of nerve growth receptors. The human promyeloid leukemic line U937 has been used to carry out this work, because of the insensitivity of human monocytes to the death mediated by α -TNF. Obtaining results opposite those observed with Fas, namely that HGH accelerates the death of these cells mediated by α -TNF, has permitted the inventors to conclude as to the inhibitory effect of HGH on the activation of NF- κ B by α -TNF, or by other cytotoxic molecules activating NF- κ B, such as daunomycin.

Thus, the present invention has for its object to provide a new method for the treatment of cancers, and more particularly malignant hemopathies and solid tumors, offering the advantage of improving both the response of the sick person to certain anti-cancer treatments and also, potentially, the general condition of the sick person.

The invention also has for its object to provide new products for the treatment of said pathologies, having both the advantage of increasing the tumoral cell response to chemotherapy, and to improve the general condition of the patients. The new products of the invention permit decreasing the activation of the NF- κ B factor by means of the compound that is used to inhibit the activation of NF- κ B, such as the

human growth hormone, which is adapted to give rise to the inhibition of the transcription of the *MDR* genes and hence a reinforcement of the cytotoxic effects of the anti-tumor agents used, with the expected result of decreasing the dosage of these anti-tumor medications.

5 The invention has for its object the use of compounds inhibiting the activation of NF- κ B, for the preparation of medications adapted for the treatment of malign hemopathies and solid tumors.

 The invention more particularly has for its object the use of NF- κ B inhibitor compounds, for the preparation of medications for the
10 prevention of the appearance or the treatment of phenomena of resistance to cytotoxic molecules used in the field of treatment of the above-mentioned pathologies, these resistance phenomena arising in patients treated with these molecules when these latter are adapted to activate NF- κ B.

15 By compounds inhibiting the activation of NF- κ B (also called NF- κ B inhibitor compounds), there is meant any compound capable of inhibiting in the cells of the organism, the activation of NF- κ B caused by the cytotoxic molecules used in the field of treatment of the above-mentioned pathologies, and hence any compound capable of inhibiting the
20 synthesis of proteins (such as P-gp) permitting the cells to eliminate the molecules before they can reach their molecular targets.

 The invention relates more particularly to the above-mentioned use of compounds inhibiting the activation of NF- κ B, in association with one or several cytotoxic molecules usable in the field

of treatment of malign hemopathies or solid tumors, said cytotoxic molecules being adapted to activate the NF- κ B factor.

Preferably, the compounds inhibiting the activation of NF- κ B used in the scope of the present invention, are compounds binding specifically to the transmembrane receptors of the cytokines of class I in the cells of the organism. Preferably, said compounds are selected from those binding to the above-mentioned receptors whose amino acid sequences of the transmembrane, intracytoplasmic and extramembrane portions have a homology of about 50% to about 70%.

10 The invention has more particularly for its object the above-mentioned use of compounds inhibiting the activation of NF- κ B as defined above, selected from growth hormone, prolactin, erythropoietin, interleukin-4, interleukin-7, G-CSF, GM-CSF, interleukin-3, interleukin-6, of human or other mammal origin.

15 Preferably, said compounds are selected from growth hormone or erythropoietin.

In this connection the invention has more particularly for its object the above-mentioned use:

- of human growth hormone, as obtained by extraction from
20 hypophysary extracts, and purification,

- or, preferably, of the recombinant human growth hormone as encoded by the nucleotide SEQ ID NO 1, or by any nucleotide sequence derived from this latter by degeneracy of the genetic code and being nevertheless capable of encoding for the human growth hormone whose

sequence in amino acids is represented by SEQ ID NO 2, said growth hormone being obtained by transformation of suitable cells with the help of vectors containing a nucleotide sequence such as described above, recovery of the recombinant protein produced by said cells, and
5 purification.

The invention also relates to the above-mentioned use, of any peptide sequence derived by addition and/or deletion and/or substitution of one or several amino acids of the sequence SEQ ID NO 2, and conserving the property of the human growth hormone of inhibiting the activation of
10 NF- κ B.

The invention has more particularly for its further object the above-mentioned use of recombinant human erythropoietin such as encoded by the nucleotide sequence SEQ ID NO 3, or by any nucleotide sequence derived from this latter by degeneracy of the genetic code and
15 being nevertheless capable of encoding for human erythropoietin, whose sequence in amino acids is represented by SEQ ID NO 4, said erythropoietin being obtained by transformation of appropriate cells with the aid of vectors containing a nucleotide sequence such as described above, recovery of the recombinant protein produced by said cells, and
20 purification.

The invention also relates to the above-mentioned use, of any peptide sequence derived by addition and/or deletion and/or substitution of one or several amino acids of the sequence SEQ ID NO 4, and preserving the property of human erythropoietin of inhibiting the activation of NF-

κB.

The invention has more particularly for its object the above-mentioned use of compounds inhibiting the activation of NF-κB as defined above, for the preparation of a medication administrable by the parenteral route (IM, IV, SC), particularly in the amount of:

- about 2 IU/kg of body weight/day in the case of human growth hormone,

- of about 150 IU/kg of body weight/day in the case of human erythropoietin.

Among the cytotoxic molecules adapted to activate the NF-κB factor used in association with said compounds inhibiting the activation of NF-κB within the scope of the present invention, can be cited:

- the cytokines,
- the anthracyclines, of which may be mentioned daunomycin, and dauxorubicin,
- the vinca-alkaloids, such as vinblastine and vincristin,
- paclitaxel (or Taxol, DCI).

Preferably, the dosage of the cytotoxic molecules used in association with said compounds is about 2 to about 5 times less than the dosage of these same molecules used alone in the scope of the treatment of malignant hemopathies and solid tumors.

By way of illustration:

- the usual daily dose of daunomycin or dauxorubicin being from 40 to 60 mg/m², the dosage of these latter in the scope of the

present invention is about 5 to 30 mg/m²,

- the usual daily dosage of vinblastine being from 5 to 7 mg/m², the dosage of this latter in the scope of the present invention is about 1 to 4 mg/m²,

5 - the usual daily dosage of vincristin being from 1 to 2 mg/m², the dosage of this latter in the scope of the present invention is about 0.1 to 1 mg/m²,

 - the usual daily dosage of taxol being about 75 mg/m², the dosage of this latter in the scope of the present invention is about 15
10 to 35 mg/m².

Among the cancers adapted to be treated in the scope of the present invention, can be cited principally:

- malignant hemopathies such as leukemias, lymphomas,
- solid tumors such as those of the ovary or the breast.

15 The invention also has for its object any product containing:

- a compound inhibiting the activity of NF- κ B such as described above, and more particularly a compound binding specifically to the transmembrane receptors of the class I cytokines as defined above,
- and a cytotoxic molecule adapted to activate the NF- κ B
20 factor,

as a combined preparation for simultaneous use, separate or prolonged over time, for the treatment of malignant hemopathies and solid tumors.

The invention also has for its object any product as defined

above, as a combined preparation for simultaneous use, separate or over time, for the prevention of the appearance, or for the treatment, of phenomena of resistance to cytotoxic molecules used in the scope of treatment of the above-mentioned pathologies, appearing in patients
5 treated with molecules when these latter are adapted to activate NF- κ B.

The invention relates more particularly to any product as defined above, characterized in that it comprises as a compound inhibiting the activation of NF- κ B, growth hormone, prolactin, erythropoietin, interleukin-4, interleukin-7, G-CSF, GM-CSF, interleukin-
10 3, interleukin-6.

Products particularly preferred in the scope of the present invention, are those comprising as a compound inhibiting the activation of NF- κ B, growth hormone or erythropoietin.

The invention has more particularly for its object any
15 product as defined above, characterized in that it comprises:

- human growth hormone obtained by extraction from hypophysary extracts, and purification,
- or, preferably, recombinant human growth hormone as described above, encoded by the nucleotide sequence SEQ ID NO 1, or by
20 any nucleotide sequence derived from this latter by degeneracy of the genetic code and being nevertheless capable of encoding for the human growth hormone whose sequence of amino acids is represented by SEQ ID NO 2, or any peptide sequence derived by addition and/or deletion and/or substitution of one or several amino acids of the sequence SEQ ID NO 2,

and preserving the property of human growth hormone to inhibit the activation of NF- κ B.

The invention also has for its object any product as defined above, characterized in that it comprises recombinant human erythropoietin such as described above, encoded by the nucleotide sequence SEQ ID NO 3, or by any nucleotide sequence derived from this latter by degeneracy of the genetic code and being nevertheless capable of encoding for human erythropoietin whose sequence in amino acids is represented by SEQ ID NO 4, or any peptide sequence derived by addition and/or deletion and/or substitution of one or several amino acids of the sequence SEQ ID NO 4, and preserving the property of human erythropoietin to inhibit the activation of NF- κ B.

The invention also relates to any product as described above, characterized in that it comprises as cytotoxic molecule adapted to activate the NF- κ B factor, any molecule selected from the following:

- cytokines,
- anthracyclines, such as daunomycin or dauxorubicin,
- vinca-alkaloids, such as vinblastine and vincristine,
- paclitaxel (or Taxol, DCI).

Products such as those defined above that are preferred in the scope of the present invention, are characterized in that they contain:

- growth hormone and daunomycin or dauxorubicin, in proportions such that their daily dosage is about 2 IU/kg of growth

hormone for about 5 to 30 mg/m² of daunomycin or dauxorubicin,

- growth hormone and vinblastine, in proportions such that their daily dosage is about 2 IU/kg of growth hormone for about 1 to 4 mg/m² of vinblastine,

5 - growth hormone and vincristine, in proportions such that their daily dosage is about 2 IU/kg of growth hormone for about 0.1 to 1 mg/m² of vincristine,

 - growth hormone and taxol, in proportions such that their daily dosage is about 2 IU/kg of growth hormone for about 15 to 35 mg/m²
10 of taxol,

 - erythropoietin and daunomycin or dauxorubicin, in proportions such that their daily dosage is about 150 IU/kg of erythropoietin for about 5 to 3 mg/m² of daunomycin or dauxorubicin,

 - erythropoietin and vinblastine, in proportions such that
15 their daily dosage is about 150 IU/kg of erythropoietin for about 1 to 4 mg/m² of vinblastine,

 - erythropoietin and vincristine, in proportions such that their daily dosage is about 150 IU/kg of erythropoietin for about 0.1 to 1 mg/m² of vincristine,

20 - erythropoietin and taxol, in proportions such that their daily dosage is about 150 IU/kg of erythropoietin for about 15 to 35 mg/m² of taxol.

The invention is illustrated with the help of the following detailed description of the *in vitro* effect of growth hormone and erythropoietin on tumoral cell lines.

1) Example No. 1:

A selection gene (*neomycin resistant*, Neo^R) and the gene encoding for human growth hormone (HGH) have been co-transfected in the human promyeloid leukemic line U937. By comparing the transfected line U937-HGH (which produces in a constituent fashion HGH at physiologic doses), either to the parent line U937, or to a line transfected with Neo^R alone, there are observed by different methodological approaches, that the U937-HGH line dies more under the effect of the *tumor necrosis factor* (-TNF). This cytokine secreted by different types of immune cells has an anti-tumor activity (Harakana, K et al., 1984, Int J Cancer, 34:263-267) and is capable of promoting the activation of NF- κ B (Baeuerle PA, Henkel T, 1994, Ann Rev Immunol, 12:141-179).

The U937-HGH cells and the U937-Neo control cells have been cultured for 48 hours in the presence of increasing concentrations of recombinant -TNF. As a result of this culture, the washed cells have been incubated in the presence of propidium iodide which is incorporated in the DNA of the dead cells. These cells are analyzed by flowing cytometry.

Figure 1 shows the increase of the incorporation of propidium iodide as a function of increasing doses of -TNF expressed in international units (IU). For the U937 cells (the mother line having

served to obtain the U937-HGH lines), with increase of the concentration of -TNF, there is observed a slight increase of the percentage of fluorescent cells (thus dead) due to the incorporation of propidium iodide (red fluorescence). This figure shows on the other hand clearly the fact that these values are much higher for the U937-HGH line, as a function of increasing doses of -TNF added to the same culture.

It is thus demonstrated that the presence in the cellular cultures of HGH produced by the U937 lines transfected with the HGH gene, increases their susceptibility to the induction of death mediated by -TNF.

2) Example No. 2:

Having reported in a previous study that HGH could intervene in the inhibition of the activation of NF- κ B mediated by lipopolysaccharides (Haeflner A et coll., 1997, J Immunol, 158:1310-1314), the inventors have studied the status of NF- κ B during stimulation of the different lines by -TNF.

Figure 2 shows the result of an analysis by gel delay. On this gel were deposited nuclear extracts from the U937-HGH and U937 cells (the mother line having served for obtaining the U937-HGH lines) subjected to different inductors including -TNF or -TNF and cycloheximide (inhibitor of protein synthesis). This experiment indicates clearly that the presence of NF- κ B in the nuclei of the U937-HGH cells, is decreased relative to the control cells.

The presence of NF- κ B is seen in lines 4 and 5, which

represent the migration of the nuclear extracts of U937 cells stimulated by -TNF, and pre-incubated, either with a cold probe muted NF- κ B which does not displace the signal (line 4), or with a cold probe NF- κ B homolog which inhibits the signal (line 5).

Figure 3 shows the result of an *enzyme immunoassay* (ELISA) carried out with the lysate of U937-HGH and U937-Neo cells transfected in a transitory manner with a plasmid containing NF- κ B sequences in the promotor of the reporter gene encoding for chloramphenicol-acetyl-transferase (CAT) (Chiao P et coll., 1994, Proc Natl Acad Sci USA, 91:28-32).

The cells are transfected by electroporation then incubated with -TNF. At the end of culturing, the cells are lysated and the activated CAT is measured by an commercial ELISA (Boehringer-Mannheim), according to the directions of the supplier.

The figure shows that the CAT activity, reflected by the presence of NF- κ B, is decreased in the U937-HGH cells relative to the control cells, after stimulation by -TNF.

The results shown in Figures 2 and 3 therefore show by two different methodological approaches, that the synthesis of NF- κ B is decreased in U937-HGH relative to the control line.

3) Example No. 3:

The use of -TNF being very difficult in human clinical work because of the adverse side effects, the inventors are interested in daunomycin. This anthracyclin used in anti-cancer therapy under the name

of Cerubidine acts by insertion in the cellular DNA sequences, thus disturbing the cellular function. Like -TNF (Baeuerle PA, Henkel T, 1994, Ann Rev Immunol, 12:141-179), daunomycin activates NF- κ B (Das KC, White CW, 1997, J Biol Chem, 272:14914-14920).

Figure 4 indicates that the U937-HGH line is also more sensitive than the control line to the mediated death by daunomycin.

4) Example 4:

To test the possibility of using the object of the present invention on non-lymphoid tumors, the inventors have used HGH to try to invert the "adriamycine resistant" phenotype of cells isolated from a human ovarian adenocarcinoma IGROV/ADR (Bénard J et coll., 1985, Cancer Res, 45:4970-4979).

As shown by Figure 5, these cells are insensitive to the toxic effect of the daunomycin added to the culture (HGH groups 0 ng/ml).

The addition of recombinant HGH (Saizen^R, Serono laboratory) renders these cells sensitive to daunomycin, with a maximum effect observed for the lowest dose of HGH used here, namely 5 ml/ml.

These result proves on the one hand that the results of aggravated mortality can be obtained as well with recombinant exogenous HGH as with the transfected lines mentioned above, and that on the other hand, the present invention can be applied to non-lymphoid solid tumors.

5) Example No. 5:

Erythropoietin (EPO), another molecule than HGH belonging to the same family of cytokines of class I, has been tested on human renal

carcinoma cells (RCC) HIEG.

4.10⁴ RCC cells have been transfected in a transitory manner with the help of an Effecten[®] kit, or with 3µg of plasmid carrying the gene encoding for EPO (RCC-EPO cells), or with 3µg of a plasmid coding for the resistance to neomycin (RCC-Neo cells) as the negative control. After 48 hours, the RCC were combined with daunomycin at two different concentrations: 0.3 and 0.6 µM. The number of surviving cells was measured 48 hours later by flow cytometry (Figure 6).

The results of Experiment 1 expressed in numbers of living cells are as follows:

	RCC-Neo	RCC-EPO
daunomycin 0µM	14745	26911
daunomycin 0.3µM	11382	3487
daunomycin 0.6µM	10179	8551

The results of Experiment 2 expressed in numbers of living cells are as follows:

	RCC-Neo	RCC-EPO
daunomycin 0µM	20150	29102
daunomycin 0.3µM	8891	2693
daunomycin 0.6µM	7001	4739

The results show that in the two different experiments (Experiments 1 and 2), the conjoint presence of daunomycin and EPO aggravates substantially the cellular mortality, with a more marked effect for the lower dose of daunomycin used.

DESCRIPTION OF THE DRAWINGS

- Figure 1: The effect of growth hormone on the mortality of cells exposed to -TNF: the percentage of the dead cells (IP+) is indicated on the ordinate, the white colonies corresponding to the cells of the strain U937-Neo, the black colonies corresponding to the cells of the strain U937-hGH; the concentrations of NFT- are indicated on the abscissa in IU/ml.

- Figure 2: The effect of growth hormone on the translocation of NF- κ B; column 1 corresponds to the control cells U937, column 2 corresponds to the U937 cells treated with -TNF + cycloheximide, column 3 corresponds to the U937 cells treated with -TNF, column 4 corresponds to the U937 cells treated with -TNF + a mutant NF- κ B probe, column 5 corresponds to the U937 cells treated with -TNF + a homologous NF- κ B probe, column 6 corresponds to the control cells U937-HGH, column 7 corresponds to the U937-HGH cells treated with -TNF + cycloheximide, column 8 corresponds to the U937-HGH cells treated with -TNF; the presence of NF- κ B is indicated by an arrow.

- Figure 3: Effect of growth hormone on the reporter activity CAT; the percentage of variation of CAT activity is indicated on the abscissa; the two left columns show the two experiments carried out on U937-Neo cells, and the two right columns represent the two independent experiments carried out on U937-HGH cells.

- Figure 4: Effect of growth hormone on apoptosis induced by daunomycin; the percentage of the dead cells (IP+) is indicated on the

ordinate, the white columns corresponding to the cells of the strain U937-Neo, the black columns corresponding to the cells of the strain U937-HGH; the indicated percentages show the increase of mortality of the cells; the concentrations of daunomycin are indicated on the abscissa in μM .

- Figure 5: Effect of growth hormone on the apoptosis of the IGROV/ADR line, induced by daunomycin; the percentage of dead cells (IP+) is indicated on the ordinate, and different columns corresponding to the different concentrations of HGH used (0, 5, 50, 500, 1000 ng/ml); the concentrations of daunomycin are indicated on the abscissa in μM .

- Figure 6: Effect of erythropoietin on the apoptosis of the human renal carcinoma line HIEG, induced by daunomycin: for each of the experiments 1 and 2, the number of living cells is indicated on the ordinate, the white columns correspond to the RCC-Neo cells, the black columns correspond to the RCC-EPO cells; the concentrations of daunomycin are indicated on the abscissa in μM .